CREATION AND ANALYSIS OF A *mut*L KNOCKOUT STRAIN OF *VIBRIO CHOLERAE*

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ABSTRACT

Many species of pathogenic bacteria have been shown to contain some form of a DNA mismatch repair system. The methyl-directed DNA mismatch repair genes (such as *mut*S, *mut*L, and *mut*H in *E. coli*) have been found to be responsible for the reduction of incorporation of foreign DNA into the *E. coli* genome (Townsend, 2003). The absence, or disruption, of these genes has been shown to increase the rate of mutations (Taddel, 1997). No studies have previously been performed on mutators in *Vibrio cholerae*. In this study, we report the development of a *mut*L deletion mutant of *Vibrio cholerae*. This knockout strain can be used to determine the function of *mut*L in *V. cholerae.* Furthermore, if the knockout produces a hypervariable strain of *V. cholerae*, that strain could be used to study natural mutations in greater detail, and at a much faster rate. The elucidation of these mutations in *V. cholerae* could change the way targets for detection, identification or even vaccines are chosen.

1. INTRODUCTION

Cholera is an ancient disease in the midst of a modern resurgence. With modern medical care, case fatality rates for cholera should be <0.25%; however, in epidemics in remote and medically underserved areas (particularly in Africa), case fatality rates often exceed 10%, and can approach 50%. Due to its ability to cause large epidemics of debilitating disease spread by food and/or water, *Vibrio cholerae* has a significant potential for weaponization. In spite of its potential for causing mass casualties it is a relatively safe organism for laboratory work and therefore is a useful model system for answering basic biological questions applicable to other biothreat agents.

V. cholerae is a diverse environmental species. Seven cholera pandemics have occurred since 1817; in each, cholera has spread rapidly from a focus in Asia to most of the known world (Pollitzer, 1959). In contrast to the diversity seen within *V. cholerae* as a whole, each cholera pandemic appears to be caused by a single, almost clonal strain, with only slight strain differences appearing as the pandemic moves across continents and through time (Momen, 1985; Wachsmuth, 1994). In 1992, a new, non-O1 *V. cholerae* strain (designated *V. cholerae* O139 Bengal), expressing a new O antigen and capsule, appeared in India and spread rapidly across the Indian subcontinent and through much of Asia (ICDDR,B 1993; Nair, 1994), supplanting "native" seventh pandemic *V. cholerae* O1 El Tor strains. These observations highlight the possibility for pandemic spread of "new," emergent strains that carry an appropriate combination of genetic material (including, potentially, novel genes or gene combinations) for a pandemic outbreak.

1.1 Mutator Strains

New biological threat agents can be produced by natural mutations, directed evolution, or via more direct molecular engineering techniques. In many cases, the engineered organism is at a disadvantage relative to wild type species in the environment, and this bears directly on its utility as a threat agent. The survival of a species is dependent upon a balance between the fidelity of its replication and the increased fitness conferred by its natural mutations. One of the methods for corrections of mismatches and mutations that occur during replication is the Dam-dependent mismatch repair system. The main components of this system, which has been found in a number of different species of bacteria, are found in the *mut*HLS pathway (Aronshtam and Marinus, 1996). When an error is found in replication, the pathway is activated and repairs are performed. Inactivation of the pathway can lead to the development of hypermutator strains that have a significantly increased rate of mutation. Such mutations would be expected to enhance adaptation of bacterial populations by increasing the supply rate of beneficial mutations during periods of strong selection; phrased another way, mutators serve as a "fast track" to generate adaptive mutations (Taddel 1997). This concept of "mutator" stains (i.e., strains with a hypermutable phenotype) is well recognized in work with *E. coli* and *Salmonella* (LeClerc, 1996). If the right environmental stressors are present, these mutator strains can lead to the production of a mutant with better survival rates or increased pathogenicity under new environmental conditions (Mekalanos, 1979; Townsend, 2003). The hypermutator phenotype is generally lost once the strain

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has adapted to the new environment, as it is deleterious to survival of the strain under normal conditions. Since an increased rate of mutation could be used to isolate strains with improved biothreat characteristics, the presence of stable hypermutator strains would be a marker for genetically engineered threat agents.

The concepts outlined above have been developed primarily in work with *E. coli* and *Salmonella*. Interestingly, in *Neisseria meningitidis*, mutations in mismatch repair have also been associated with a phenotype demonstrating high-frequency phase variation (Richardson, 2001). In a recent review, Claverys and colleagues (Claverys, 2000) have highlighted the variation between the *E. coli*/*Salmonella* SOS/*mut* system and that seen in pneumococci. In contrast to *E. coli*, pneumococci become competent during exponential growth, accepting any single-stranded DNA, with development of competence dependent on cell density. The pneumococci mismatch repair system Hex can reduce transformation frequencies for point mutations in homologous DNA, but is unable to prevent interspecies transformation. Finally, the system of stress-induced mutagenesis (i.e., the SOS response) seen in *E. coli* is not present in pneumococci. This results in what Claverys calls "programmed variability," designed to favor interspecific exchanges in the whole population, rather than the "random," strain-dependent variability seen in *E. coli*. This, in turn, leads to the hypothesis that the success of *S. pneumoniae* as a human pathogen is related to an inherent "genetic plasticity," which favors adaptation to environmental changes. No studies have been done on mutators in *V. cholerae*. However, based on the annotation of the *V. cholerae* N16961 genome sequence, *V. cholerae* appears to have the intact *mut* mismatch repair (MMR) system, including *mutH*, *mutL*, *mutS*, *mutT*, *mutU*, and *mutY* (Friedhoff 2002). The apparent increase in recombination rates within the species raises the possibility that *V. cholerae* also has elements of "programmed variability," increasing its ability to adapt to changing environments and its success as a human pathogen.

To understand evolutionary change in cholera, we need to have an understanding of mechanisms involved in this change, and the contributions that they make to the mutation rates of the species as a whole. Therefore, producing a knockout strain of *Vibrio cholerae*, containing an internal deletion within the *mut*L gene, would help to determine the effect of the *mut*HLS pathway on *Vibrio* evolution.

2. MATERIALS AND METHODS

2.1 Bacterial strains and media

Bacteria were stored in LB broth and 10% glycerol at -80°C, and grown in LB broth, on LB and LB without NaCl agar. *E. coli* XL1 Blue cells (Stratagene, La Jolla, CA) were the host for transformation of the pBluescript plasmids (Strategene) used. *E. coli*SM17λpir (University of Maryland Baltimore [UMB], Baltimore, MD) was used at the host for transformation of the pCVD442 plasmid (UMB). Polymyxin B resistant *V. cholerae* N16961 (UMB) was used as the host strain for the crosses performed. Carbenicillin (100µg/ml), polymyxin B (50U/ml) and sucrose (10%) (SIGMA, St. Louis, MO) were added to the media as appropriate.

Competent cells were made by growing the cultures to mid-logarithmic phase, chilling on ice for 30 minutes, washing three times with distilled water, and resuspending them in 10% glycerol. Electroporation was performed in chilled, 0.1-cm cuvettes using the E. coli Pulser (Bio-Rad, Richmond, CA) at 1.88 kV.

2.2 Molecular biology

Primers were designed using Primer Express Software (Applied Biosystems Inc. [ABI], Foster City, CA) and analyzed using the web-based OligoAnalyzer 3.0 software (Integrated DNA Technologies, http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx) PCR, on an ABI 9600 (ABI) agarose gel electrophoresis, restriction enzyme digests, and ligations were performed using standard techniques (Sambrook, 1989). Extractions and purifications were performed using the appropriate QIAgen Kit and corresponding protocols (QIAGEN, Inc., Valencia, CA).

3.0 RESULTS

3.1 Amplification of genes

The genome sequence of *Vibrio cholerae* was analyzed for the location, orientation and open reading frame (ORF) of the *mut*L gene. Primers were designed to amplify a truncated version of the *mut*L gene (Δ*mut*L) that did not disrupt the original orientation and ORF (Table 1). By keeping the ORF intact, any manipulations using the truncated *mut*L gene could attribute phenotypic changes to the disruption of the mutator gene, and not any of the surrounding genes. Using primers 1 and 2, listed in Table 1, a 602bp fragment was created that contains part of the gene VC0344, which encodes *N-acetylmuramoyl-L-alanine amidase*, the leading piece of *mut*L, as well as the unique restriction sites for *Kpn*I, *Xma*I, and *Hind*III (*mut*1). Using primers 3 and 4, listed in Table 1, a 596bp fragment was created that contains part of the gene VC0346, which encodes *delta(2)-isopentenylpyro-phosphate transferase*, the end piece of *mut*L, as well as the unique restriction sites for *Xba*I, *Xma*I, and *Hind*III (*mut*2), (Figure 1A). These amplified fragments were separated by gel electrophoresis, extracted and purified.

Table 1. Primers used in these experiments. Underlined sequence indicates the unique restriction sites added.

Primer name	Sequence	Start site
1	5'-TTAGGTACCCCCGGGGGTAGTGGTGATAACC-3'	366523
2	5'-TTAAAGCTTATTGCTGCCGGATCTGTTATCTTACGC-3'	367043
3	5'-ACCAAGCTTTGACTACTGAAGCCGGTCTTTCT-3'	368734
4	5'-ATTTCTAGACCCGGGCCGAGGTAAAGCTCA-3'	369315

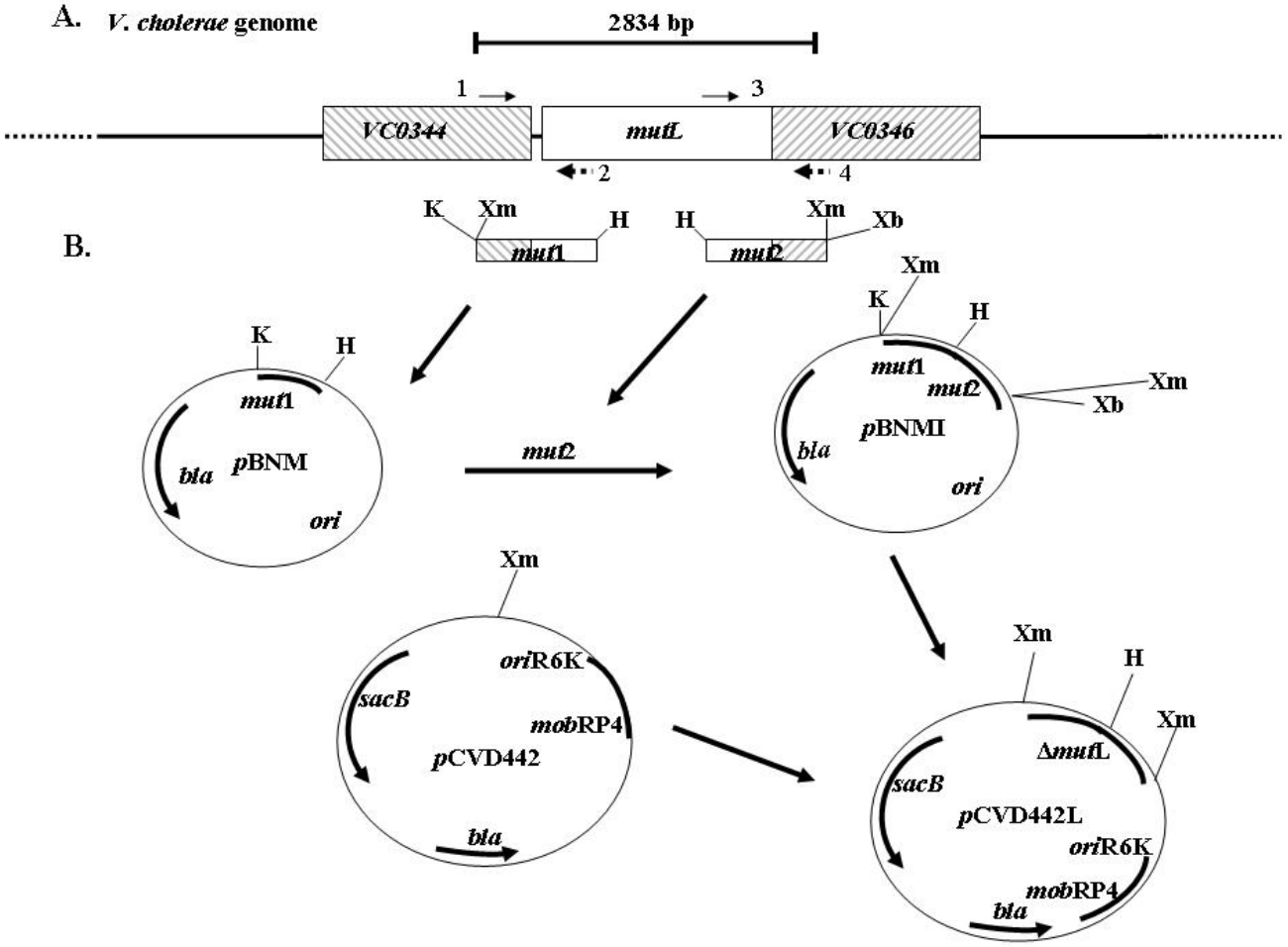

Figure 1. **A**. Schematic of part of the *V. cholerae* N16961 genome used for cloning *mut*L. Shaded boxes indicate the genes flanking *mut*L. Solid arrows indicate placement of forward primers, dotted arrows indicate placement of reverse primers. Numbers correspond to primer sequences located in Table 1. K: *Kpn*I, H: *Hind*III, Xm: *Xma*I, Xb: *Xba*I. Bar above genome indicates the size of the fragment produced in the intact genome, using primers 1 and 4 from Table 1. Genes are not drawn to scale.

B. Scheme for the construction of suicide vector pCVD442L, which contains a truncated version of *mut*L from *Vibrio cholerae* N16961. Plasmid pBNM was created by amplifying a fragment of the *V. cholerae* genome containing the 3'end of VC0344 and the 5'end of *mut*L, digesting both the fragment and pBluescript with KpnI and HindIII, joining the two with T4 DNA ligase, and selecting for ampicillin resistant colonies in *E. coli* XL1Blue cells. Similarly, pBNMI was created by amplifying a fragment of the *V. cholerae* genome containing the 3'end of *mut*L and the 5'end of VC0346, digesting both the fragment and pBMN with *Hind*III and *Xba*I, joining the two with T4 DNA ligase, and selecting for ampicillin resistant colonies in XL1Blue. The Δ*mut*L fragment was created by cutting pBMNI with *Xma*I and joining the 1.2-kb fragment with the *Xma*I-digested plasmid pCVD442, which contains the *sac*B gene of *B. subtilis* and the *bla* gene. The resulting suicide vector was selected by ampicillin resistance in *E. coli* SM17λpir cells. Plasmids are not drawn to scale.

3.2 Construction of intermediates pBNM and pBNMI

Plasmid pBNM was created by digestion of both the purified *mut*1 fragment and pBluescript with *Kpn*I and *Hind*III, separation by gel electrophoresis, extraction, purification and joining the two with T4 DNA ligase (Figure 1B). Competent XL1 Blue cells were transformed with the ligated reactions and transformants were selected with carbenicillin. Success of the reaction was verified by plasmid extraction, digestion and the visualization of a 602bp band after gel electrophoresis.

Similarly, plasmid pBNMI was created by digestion of both the purtified *mut*2 fragment and pBNM with *Hind*III and *Xba*I, separation by gel electrophoresis, extraction, purification and joining the two with T4 DNA ligase (Figure 1B). Competent XL1 Blue cells were transformed with the ligated reactions and transformants were selected with carbenicillin. Success of the reaction was verified by plasmid extraction, digestion and the visualization of a 596bp band after gel electrophoresis.

3.3 Construction of pCVD442L

The suicide vector, pCVD442L, was created by the adaptation of the vector described by Donnenberg and Kaper (1991). Plasmid pCVD442 contains the *bla* gene, which allows for the selection of antibiotic resistance. This plasmid also contains the *sac*B gene of *B. subtilis*, the locus that encodes the enzyme levan sucrase, which is toxic for gram negative organisms only in the presence of sucrose. This conditionally lethal phenotype can lead to the selection of colonies that have undergone an allelic exchange by homolgous recombination. Furthermore, this plasmid contains the origin of plasmid R6K and the *mob* gene, which ensures that its replication is dependent upon π, the *pir* gene product (Donnenberg, 1991). In this study, the suicide vector pCVD442L was created by the digestion of both pBNMI and pCVD442 with *Xma*I and joining the 1.2kb Δ*mut*L fragment with the suicide vector using T4 DNA ligase (Figure 1B). Competent SM17λpir cells were transformed with the ligated reactions and transformants were selected with carbenicillin. Success of the reaction was verified by plasmid extraction, digestion and the visualization of a 1.2kb band after gel electrophoresis (Figure 2).

3.4 Construction of knockout *V. cholerae* strain

Homologous recombination was set up with *E. coli* SM17λpir containing pCVD442L as the donor strain and *V. cholerae* N16961 as the recipient strain. Various ratios of the donor and recipient, used to ensure the optimal growth of the bacteria, were pipetted onto a sterile 0.2 μm filter on top of an LB agar plate. These mixed cultures were grown without selection for six hours, and the filters were then placed into sterile phosphate buffered saline (PBS). Serial dilutions were spread onto LB plates containing polymyxin B and carbenicillin. Colonies that grew on these plates, under both selections, should be *V. cholera* that has undergone a single cross-over with the pCVD442L plasmid.

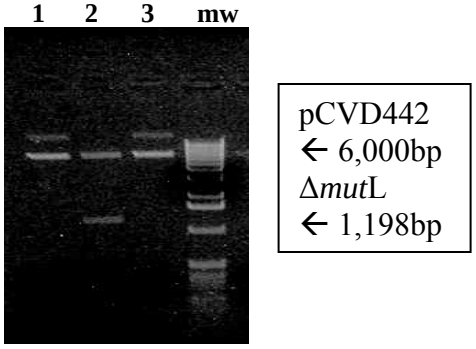

Figure 2. Agarose gel of pCVD442L digested with *Xma*I. Lanes 1-3: miniprep DNA; mw, TrackIt (Invitrogen) 1 Kb DNA Ladder (bp of bands: 12,216, 11,198, 10,180, 9,162, 8,144, 7,125, 6,108, 5090, 4072, 3054, 2036, 1636 [bright], 1018, 506/517). The miniprep DNA in lane 2 contains both pCVD442 (6,000bp) and Δ*mut*L (1,198 bp)

Isolates found to be both polymyxin B and carbenicillin resistant were grown without selection to late logarithmic phase and spread on LB plates without NaCl but containing 10% sucrose. These same colonies were also split and grown on LB plates containing polymyxin B and carbenicillin for verification of their resistance. Colonies that grew in both places were then patched onto both sucrose plates and carbenicillin plates. Colonies that are resistant to carbenicillin would contain a single cross-over event, retaining the resistant plasmid within the genome. Colonies that are unable to grow on the carbenicillin plates, but do grow on the sucrose plates would have undergone a double cross-over in the *Vibrio* genome, resulting in the Δ*mut*L gene (Figure 3). If the colony had reverted back to the original genome, it would not be resistant to carbenicillin or be able to utilize sucrose well.

3.5 Verification of double cross-over event

Twelve colonies were found to be carbenicillin sensitive and able to utilize sucrose. In order to determine if the colonies contained the Δ*mut*L gene, PCR was performed using primers 1 and 4 from Table 1. If the *Vibrio* underwent a double cross-over event, those primers would produce an amplicon of approximately 1.2Kb. However, if the *Vibrio* reverted back to the original genome, with another single cross-over, those primers would produce a 2880 bp amplicon. There were nine of the twelve colonies that produced 1.2Kb bands (Δ*mut*L) when run on an agarose gel (Figure 4), the remaining three colonies produced 2.8Kb bands (*mut*L).

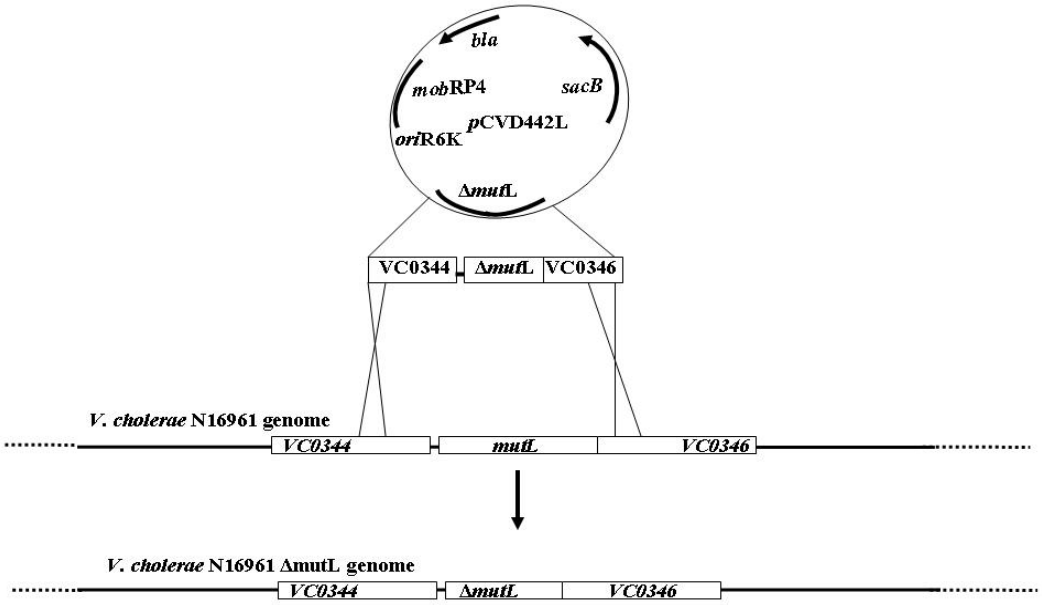

Figure 3. Schematic of cross-over events between pCVD442L and *V. cholerae*.

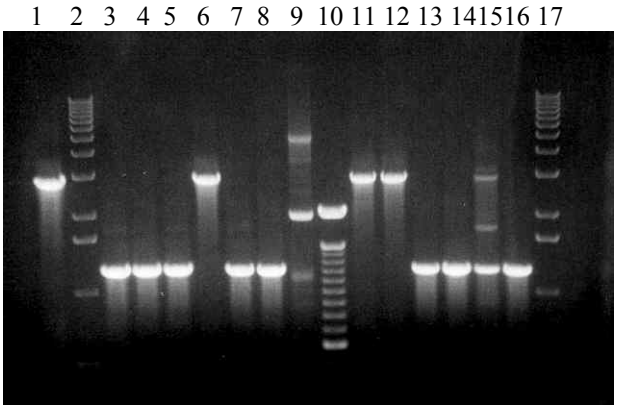

Figure 4. Agarose gel of amplicons from *V. cholerae* PCR. Controls: Lane 1, *V. cholerae* N16961; lanes 2 and 17, TrackIt 1Kb DNA ladder (see legend of Figure 2 for bands); lane 9, pCVD442L; lane 10, 100 bp DNA ladder (GibcoBRL) 10 fragments between 600 and 1500 bp in multiples of 100 bp, with an additional fragment at 2072 bp; lane 18, NTC. All other lanes contain colonies that were carbenicillin sensitive and able to utilize sucrose.

CONCLUSIONS

The *Vibrio cholerae* colonies that were both sensitive to carbenicillin and able to utilize sucrose were believed to contain the Δ*mut*L gene. This was confirmed by PCR, which produced the appropriate size amplicon. Further testing is needed to determine the exact sequence in the area of the gene of interest. Primers have been designed which would produce an amplicon within the truncated area. If such an amplicon is produced, than the *mut*L gene would be intact within that colony's genome. Once a knockout strain of *Vibrio cholerae* is established, testing of this strain can be performed.

Most bio-detection and identification assays rely on the use of either antibodies, which recognize surface components, or PCR, which recognizes specific genes within a species. By characterizing a mutator strain, detection or identification assays could be developed that would recognize global rather than specific genes. These global genes could help find multiple threats with the same assay, or they could help to determine highly mutable regions of a genome which should be avoided as targets for assays. The elucidation of these genes in *Vibrio cholerae* could open the way to new methods for detection, identification or even how vaccines are chosen. Furthermore, this research could help to study potential biological agent variation due to genetic drift by providing important information about DNA repair systems in *V. cholerae*. Discerning these processes will help us recognize how new serogroups emerge in natural settings as well as during laboratory manipulations. This in turn will improve our ability to develop sensitive detection reagents and provide information vital to vaccine development. Understanding recombination and horizontal gene transfer also has implications for the survival of virulence genes in the environment and the potential

transfer of these genes leading to the emergence of new pathogens.

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